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Isolation and Characterization of Propoxur-Degrading Bacteria, Brucella pseudintermedia LED 6 from a Pineapple Plantation in Lampung

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ABSTRACT

Propoxur is a non-systemic carbamate insecticide that is widely used in Indonesia to control insect pests. However, its persistence and toxicity pose environmental concerns. Bioremediation with bacteria is a viable method for mitigating the detrimental effects of propoxur residues. The goal of this work was to isolate and characterize bacterial strains that can degrade propoxur. Isolation by enrichment culture procedures, utilizing propoxur as the sole carbon source. The isolates' morphological and physiological features were examined, and their degradation potential was determined. Six bacterial samples were isolated from a pineapple plantation in Lampung, Indonesia, and one strain, known as LED 6, showed great potential for propoxur breakdown. Molecular identification with 16S rRNA gene sequencing identified the isolate as Brucella pseudintermedia. Growth characterisation revealed that the isolate performed best at 28 °C and pH 7. After 72 hours of incubation with 500 ppm propoxur, LED 6 had deteriorated around 26% of the starting concentration.

Keywords: bioremediation, Brucella pseudintermedia, pineapple, propoxur

INTRODUCTION

Pesticides are very poisonous chemical compounds that are widely employed in agriculture to control pests and diseases. Pesticides are also used in households to kill mosquitos, cockroaches, and other nuisance insects (Sun and Lee 2003). Although pesticide use can increase food production to satisfy rising demand, synthetic pesticides have negative consequences for humans, animals, and the environment (Yadav *et al.* 2021). Propoxur (2-isopropoxyphenyl-N-methylcarbamate) is one of the environmentally toxic active components of insecticides. It is an active pesticide chemical used to control insects, specifically mosquitoes (Thomson 1985). It works by inhibiting acetylcholinesterase in the neurological system of animals, and its presence not only poisons target organisms but also harms non-target organisms (Fahmy *et al*. 1970; Krechniak and Foss 1982; Yadav *et al*. 2010. Kuseske *et al*. (1974) found that propoxur at a concentration of 0.5 g/L can impede the nitrification process. Excessive and uncontrolled pesticide use poses poisoning risks that can result in a variety of negative outcomes, including pesticide residue accumulation in agricultural products, environmental contamination, animal poisoning, and even potentially fatal human poisoning (World Health Organization [WHO] 2005; Jae *et al*. 2014). Bioremediation is one strategy for mitigating the impact of propoxur.

Bioremediation is a method for reducing the negative effects of pesticide residues or active chemicals (Shin 2012). It combats pesticide contamination by exploiting microbial activity, which includes bacteria, fungi, and algae (Alexander 1994; Hussain *et al*. 2009; Leja and Lewandowicz 2010. Several bacterial isolates capable of decomposing propoxur have been discovered from various environmental sources, including rice fields in Indonesia (Imamuddin *et al*. 2015; Sahlan *et al*. 2014). Ou *et al*. (1992) discovered that *Arthrobacter* sp. can breakdown propoxur and use the resulting product, 2-isopropoxyphenol, as a carbon source. According to Kamanavalli and Ninnekar (2000), *Pseudomonas* sp. may hydrolyze propoxur and use the resulting methylamine as a carbon and nitrogen source. Topp *et al.* (1993) discovered that strain ER2, which possesses a plasmid similar to that of *Achromobacter* sp., can hydrolyze *N*-methylcarbamate compounds, including propoxur. Furthermore, *Neisseria subflava*, *Staphylococcus aureus* (Anusha *et al*. 2009), *Pseudaminobacter* sp., and *Nocardioides* sp. (Kim *et al*. 2017) have been demonstrated to break down propoxur into phenol residues and simple chemicals.

Given the environmental risks posed by pesticides, this study sought extract bacteria capable of adapting to propoxur medium. The bacteria were discovered in waste material from a pineapple farm run by PT Great Giant Pineapple, which uses propoxur to combat insect

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pests. Bacteria that can thrive on propoxur-containing media could be used as inoculum sources for pesticide bioremediation. Microbial bioremediation of pesticide residues is essential since it is applicable in contaminated agricultural and plantation fields and can be used in conjunction with compost applications.

METHODS

Location and Time of Research

Sampling was done at the sewage pond to soak pineapple seedlings at PT Great Giant Pineapple in Lampung. The study was conducted in the Microbial Nutrition and Plant Protection Research Group Laboratory, Applied Microbiology Research Centre, National Research and Innovation Agency (BRIN), Cibinong, Bogor Regency, West Java Province.

Tools and Materials

An electronic balance, autoclave, laminar air flow, petri dishes, Bunsen burner, microscope, shaker, vortex mixer, magnetic stirrer, spectrophotometer, centrifuge, UV transluminator, micropipettes, Erlenmeyer flasks, inoculating loops, alcohol sprayer, cuvettes, stirring rods, L-shaped spreaders, oven, digital hot plate, camera, and stationery were used in this study.

The study's materials included 70% alcohol, distilled water, cotton, aluminum foil, wrapping plastic, tissue paper, HDPE plastic, spirit, pipette tips, NB (nutrient broth) media, waste pond water, mineral media (based on Park and Ka 2003), technical grade (TG) and professional analysis (PA) propoxur.

Isolation and Selection of Bacteria

Samples from the waste pond were collected in 100 mL quantities and deposited in 250 mL Erlenmeyer flasks, which were then supplemented with 0.05 g and 0.1 g of solid propoxur to achieve concentrations of 500 ppm and 1000 ppm of technical grade (TG) propoxur. These were acclimated for about a month. The acclimated colonies were injected onto mineral media, then serially diluted. Each dilution level was tested in duplicate. Bacterial isolation using the spread plate method was performed aseptically on mineral media supplemented with propoxur in a laminar airflow cabinet. Bacterial cultures were cultured for 5−7 d at 28 °C. Colonies were then purified by subculturing single colonies on mineral media with 300 and 500 ppm propoxur, which corresponded to the concentrations employed during the original isolation.

Bacterial Characterization

Purified bacterial isolates were examined morphologically. Pathogenicity tests were performed to ensure that the isolates were not pathogenic (Hikmawati *et al*. 2019).

Identification of Potential Pesticide-Degrading Bacteria

Molecular analysis of the 16S rRNA gene with universal primers 27F (5′-AGAGTTTGATCCTGGTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACT-3′) was used to identify selected bacterial isolates at the species level (Weisburg et al. 1991). The sequencing findings were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) via the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

The isolate's phylogenetic tree was analyzed using MEGA 6 software (Tamura et al. 2013). CLUSTAL W (Thompson et al. 1997) was used to align the sequences, and the phylogenetic tree was built using the Maximum Likelihood approach (Hasegawa *et al.* 1991) with 1000 bootstrap replications (Felsenstein 1985). According to Lapage *et al*. (1990), a type of strain is the original or reference strain used to characterize species and subspecies, and it is crucial in estimating the evolutionary distance of new species (Kyrpides *et al*. 2014).

Propoxur Degradation Assay

The degradation assay was carried out by inoculating chosen isolates from solid medium into 100 mL of mineral broth in 250 mL Erlenmeyer flasks containing 100, 300, and 500 ppm propoxur. The cultures were incubated in a shaker. Bacterial growth curves were calculated by measuring optical density (OD) at 436 nm with a UV-VIS spectrophotometer (Dewi *et al.* 2015). Before measuring the concentration of propoxur, the culture media was sampled and centrifuged to eliminate bacterial cells.

Data Collection and Analysis

Propoxur concentrations were determined using high-performance liquid chromatography (HPLC) in accordance with WHO (1999) procedures. Samples were centrifuged at 12,000 rpm for 10 min and filtered through 0.2 µm Millipore filters to remove leftover pellets. The samples were loaded into the HPLC apparatus with a mobile phase of acetonitrile:distilled water (60:40). The retention times of chromatographic peaks were compared to those of the propoxur standard. Propoxur concentration was determined using the area ratio between the sample and standard peaks. Peaks outside the normal range were considered to be degradation intermediates such methylamine and 2-isopropoxyphenol (Kamanavalli and Ninnekar 2000). They were tested against previously developed standards of PA-grade propoxur ranging in concentration from 0 to 1000 ppm.

RESULTS AND DISCUSSION

Isolation and Selection of Pesticide-Degrading Isolates

Isolation on mineral media with the spread plate technique produced six colonies with unique morphological characteristics (Figure 1). The addition of propoxur to the mineral media limited microbial growth, allowing only resistant bacteria capable of using propoxur as a nutrition source to proliferate. The isolated bacterial colonies were streaked onto six different Petri dishes. After seven days of incubation, the colonies were not completely pure. Several bacteria were discovered on a single petri plate, and purification was accomplished by subculturing specific colonies. Teng et al. (2019) observed morphological characteristics such as shape, margin, elevation, optical qualities, and pigmentation using the method they developed. Table 1 illustrates colony morphology.

Pathogenicity Testing

Pathogenicity was determined using the hemolysis assay on blood agar media. The streak plate method was used, and the presence of clear zones around bacterial colonies was observed. The results showed that LED 3 had beta-hemolysis, which is total hemolysis and indicates pathogenicity; LED 1 had alpha-hemolysis, which is partial hemolysis and is also considered harmful. The remaining four isolates (LED 2, LED 4, LED 5, and LED 6) showed gamma-hemolysis, indicating nonpathogenicity. The detailed test results are provided in Figure 2 and Table 2.

The clear zone suggests a positive hemolytic reaction in which bacteria can lyse red blood cells, implying pathogenic potential for animals or humans. Hemolysis is categorized into three types: beta (β), which shows complete hemolysis with a clear zone; alpha (α), which indicates partial hemolysis with a greenish discoloration; and gamma (γ), which indicates no hemolysis or medium change (Oktafiyanto *et al.* 2018).

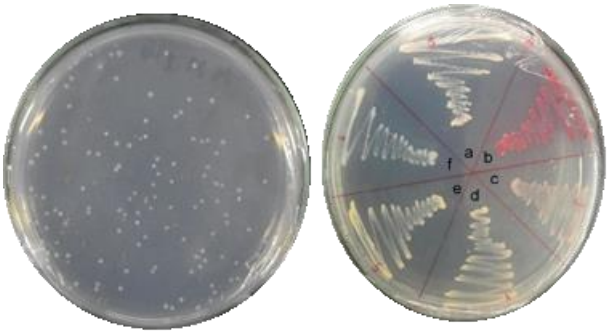

Figure 1 Left: Photo of isolates before purification, Right: Photo of isolates after purification (a): LED isolate 1, (b) LED isolate 2, (c) LED isolate 3, (d) LED isolate 4, (e): LED isolate 5, (f) LED isolate 6.

Table 1 Morphology of potential propoxur-degrading bacteria

Isolate	Characteristics of isolate colonies				
	Shape	Margin	Elevation	Optical	Pigment
LED 1	Circular	Entire	Convex	Transparent	White
LED 2	Circular	Entire	Convex	Opaque	Merah
LED 3	Circular	Entire	Pulvinate	Opaque	Orange
LED 4	Circular	Entire	Convex	Opaque	Yellow
LED 5	Circular	Filamentous	Convex	Opaque	Yellow
LED 6	Irregular	Entire	Umbonate	Transculent	Yellow

Figure 2 Hemolysis testing on blood agar media LED 1 shows alpha hemolysis (α); LED 3 shows beta hemolysis (β); LED 2, LED 4, LED 5, LED 6 shows gamma hemolysis (γ)

Growth Testing in Liquid Media Containing Propoxur

Based on the hemolysis test, four isolates were chosen: LED 2, LED 4, LED 5, and LED 6. These were cultivated in mineral broth to determine their growth under carbon-limited conditions, and optical density (OD) was measured at 436 nm. Bartha *et al.* (1967) discovered that pesticides inhibit oxidative phosphorylation, which is required for ATP generation. Propoxur is thought to block this mechanism, resulting in decreased ATP levels as the compound's concentration increases.

According to the results of the OD measurements in Figure 3, the isolate grows quicker when the propoxur concentration is low. The highest value of LED isolate 2 with an OD value of 100 ppm treatment for 72 h produced 1.63 nm; LED isolate 4 with an OD value of 100 ppm treatment for 72 h produced 3.56 nm; LED isolate 5 with an OD value of 300 ppm treatment for 108 h produced 0.25 nm; and LED isolate 6 with an OD value of 300 ppm treatment for 96 h generated 4.69 nm. Thus, isolates LED 2, LED 4, and LED 5 grew much slower and had much lower OD than LED 6. Therefore, LED 6 was selected for additional testing, including growing condition optimization (temperature and pH) and molecular identification. Molecular research confirmed LED 6's identify as *Brucella pseudintermedia*.

Molecular Identification of *Brucella pseudintermedia*

The selected isolate's DNA amplification produced a distinct and thick band on the agarose gel following PCR, indicating a fragment length of around 1510 bp (Figure 4). The bacterium was identified as Brucella pseudintermedia after sequencing the 16S rRNA gene, which shared 99.85% of its sequence with known sequences in the NCBI database. B. pseudintermedia belongs to the genus Brucella and can be discovered in plant, soil, and wastewater samples. It is catalase-positive, aerobic, Gram-negative, and thrives at pH 5−9 at temperatures ranging from 25 to 45°C. Its synonym is Ochrobactrum pseudintermedium (Teyssier et al. 2007). Ochrobactrum species have been extensively studied for their ability to bioremediate contaminants such as malathion (Verma *et al.* 2021), glyphosate (Masotti *et al.* 2021), and petroleum hydrocarbons (Al-Mur *et al.* 2021).

Based on a BLAST (Basic Local Search Alignment Tool) search on the NCBI (National Center for Biotechnology Information) website, the level of sequence similarity was derived from the Brucella genus sequence and confirmed by the level of sequence similarity based on the percentage of query cover, with Brucella pseudintermedia having the highest percent identity (Table 3). Query cover is the proportion of sequences in the alignment that match the sequence in the GenBank database. Meanwhile,

Table 2 Results of bacterial pathogenicity tests on blood agar

Isolate	Hemolysis test
LED 1	α
LED 2	γ
LED 3	β
LED 4	γ
LED 5	γ
LED 6	γ

Remarks: Alpha (α): isolate is pathogenic; beta (β): isolate is pathogenic; gamma (γ): isolate is not pathogenic to living things

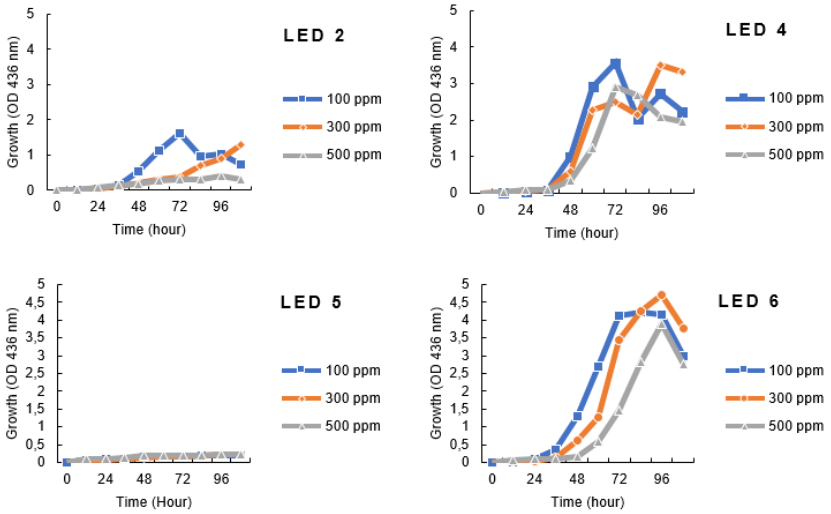

Figure 3 Growth curve of potential propoxur-degrading bacterial isolates in mineral liquid media in 250 mL Erlenmeyer flasks shaken at 100 rpm at 28°C; measurement of bacterial isolates using a spectrophotometer with a wavelength of 436 nm

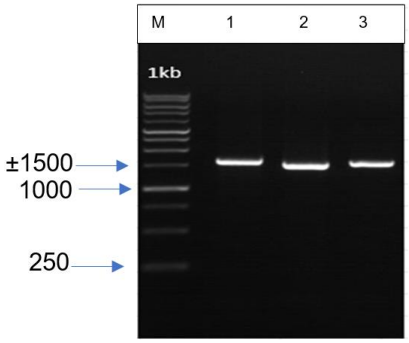

Figure 4 PCR of DNA isolate LED 6.

Table 3 BLAST sequencing of potential pesticide degrading isolates at NCBI

Isolate names in GenBank	Query cover (%)	Identity (%)	*E*–value	Accession number
Brucella pseudintermedia	100	99.85	0.0	MK351298.1
Brucella inopinata	100	98.35	0.0	MT186162.1
Brucella canis	100	97.74	0.0	OR905739.1
Brucella daejeonensis	100	97.06	0.0	MN258541.1
Brucella ovis	100	97.06	0.0	MN990902.1
Phyllobacterium phragmitis	98	95.96	0.0	NR_164937.1

percent identity represents the percentage of identity or match between the query sequence and the database sequence. The E-value is an estimated value that offers a statistically significant metric for the two sequences compared with BLAST, considering the number of matching residues and the entire length of the alignment (Newell *et al.* 2013; Ainiyah *et al.* 2020).

Phylogenetic analysis (Figure 5) employed reference sequences to assess evolutionary relationships and was represented by a phylogenetic tree (Dharmayanti 2011). According to Hillis et al. (1993), phylogenetic trees contain bootstrap values that evaluate the confidence and stability of tree branches. Bootstrap values higher than 50% are regarded statistically significant (Kress 2002). In this investigation, B. pseudintermedia had 100% similarity with the tested strain, while Phyllobacterium phragmitis formed a distinct clade.

Degradation Assay of B. pseudintermedia

Figure 6 depicts further tests undertaken to identify the ideal temperature and pH for the growth of B. pseudintermedia. Ananda (2012) stated that the ideal pH for pesticide biodegradation is 6.9. Results showed that LED 6 grew best at 28°C and pH 7, with statistically significant changes in growth.

The propoxur degradation assay by B. pseudintermedia (Table 4) showed a decrease in propoxur levels, beginning at 48 h and continuing up to 72 h. Bacterial growth attained an optical density (OD) of 1.75 nm, followed by a 26% decrease in propoxur concentration, measured at a wavelength of 436 nm with an initial propoxur concentration of 500 ppm. B. pseudintermedia uses propoxur as a carbon source to maintain its growth, as evidenced by a positive link between bacterial proliferation and breakdown rate. Anusha et al. (2009) found that Neisseria subflava and Staphylococcus aureus can degrade propoxur at a concentration of 200 ppm. This study shows that B. pseudintermedia may effectively degrade propoxur at greater concentrations (500 ppm).

CONCLUSION

Isolate LED 6 outperformed the other six bacterial isolates in the presence of 300 ppm propoxur. LED 6 significantly reduced propoxur levels by 26% after 72 h of incubation. The isolate grew optimally at pH 7 and 28 °C (mesophilic temperature). LED 6 has been identified as *Brucella pseudintermedia* using molecular analysis.

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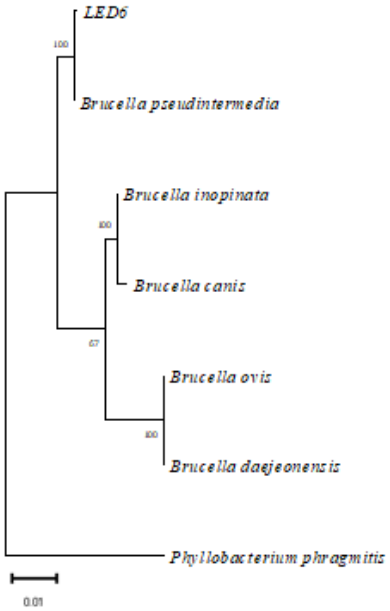

Figure 5 Phylogenetic tree of LED isolate 6.

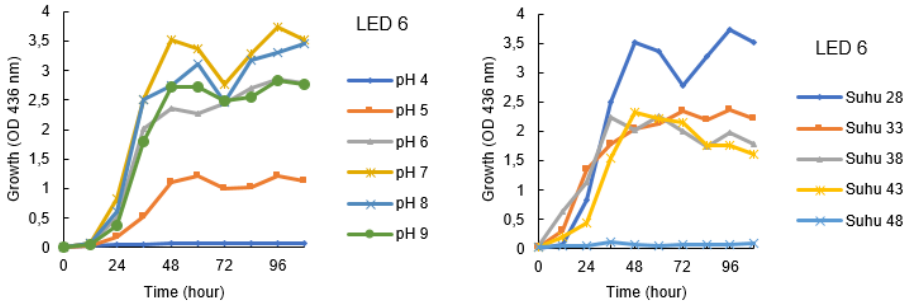

Figure 6 Characterization of Brucella pseudintermedia bacterial growth at different pH and temperatures

Table 4 Result of *Brucella pseudintermedia* bacteria on propoxur degradation

Incubation time (h)	Propoxur concentration (ppm)	Area	OD
0	436.64	1309917	0.05
24	494.57	1483720	0.62
48	432.71	1298131	0.70
72	366.89	1100680	1.75

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